Salivary Gland Protein Expression after Bion-M1 and Space Shuttle STS-135 Missions

Maija Mednieks¹, Aditi Khatri¹, and Arthur R. Hand²

¹Department of Oral Health and Diagnostic Sciences; ²Department of Craniofacial Sciences, University of Connecticut School of Dental Medicine, Farmington, CT

ABSTRACT

Secretory proteins produced by salivary glands are stored in granules and released into saliva. Rodent salivary glands are a reliable experimental model because they are morphologically and functionally similar to those of humans. To determine if the effects of microgravity on secretory proteins are increased on extended flights, their expression in mouse parotid glands, morphological, immunocytochemical, and biochemical/molecular methods were employed. Acinar cells of STS-135 (13 day) and Bion-M1 (30 day) flight animals showed an increase of autophagy and apoptosis, while duct cells contained vacuoles with endocytosed proteins. In STS-135, decreases were seen in the regulatory subunit of type II protein kinase A (RII) by Western blotting, and demilune cell and parotid protein (DCPP) and α-amylase (p<0.01) by immunogold labeling, while proline-rich proteins (PRPs, p<0.001) and parotid secretory protein (PSP, p<0.05) were increased. These results suggest microgravity effects on secretion are function-dependent. Microarray analyses showed significant changes in the expression of a number of genes, including components of the cyclic-3',5'-adenosine monophosphate (cyclic AMP) signaling pathway. Compared to habitat ground controls, mice from both flights exhibited altered expression of cyclic AMP-specific phosphodiesterases, adenylate cyclase isoforms, and several A-kinase anchoring proteins. Bion-M1 flight mice showed increases in gene expression for lysozyme and amylase, a decrease in PRPs, and RII expression was unchanged from control values. Secretory protein expression is altered by travel in space, representing a reversible adjustment to microgravity conditions. Ultimately, the goal is to develop a test kit using saliva — an easily obtained body fluid — to assess the physiologic effects of travel in space.

INTRODUCTION

Background

Understanding the effects of weightlessness during travel in space is important in order to institute countermeasures if the findings are
detrimental. Catecholamine hormone-controlled cellular events are altered during spaceflight (Mednieks et al., 1998; Mednieks et al., 2000; Mednieks et al., 2014), indicating an environmental effect or a response to microgravity. In salivary glands, β-adrenergically regulated responses modify exocytosis and protein secretion (Bdolah and Schramm, 1965; Butcher and Putney, 1980; Mednieks and Hand, 1982; Horio et al., 1984). Protein expression, mediated via cyclic AMP pathways, is particularly affected and may serve as an index of physiological or disease-related changes (Gold et al., 2013) [e.g., in diabetes (Szczepanski et al., 1998; Mednieks et al., 2009)], as well as responses to mechanical (Burke et al., 2002) or environmental stimuli (Beavo and Brunton, 2002).

Figure 1 is a diagram of the components of exocytotic stimulus pathways with the β-adrenergic receptor (βAR) activating adenylate cyclase (AC), formation of cyclic AMP [with phosphodiesterase (PDE) to lower intracellular levels], activation of cyclic AMP-dependent protein kinase (PKA) by subunit dissociation, and the eventual nuclear reactivity of the type II regulatory subunit (RII) via the action of DNA binding A-kinase anchor proteins (AKAPs) (Dodge et al., 2001; Herberg et al., 2000). The metabolic interplay of these components provides an internal stability to environmental changes at the cellular level of organization. The individual molecular components of this pathway are well-studied (Daniel et al., 1998; Gold et al., 2013) and provide a series of biomarkers for biochemically measuring stress responses. Serum levels of these components are difficult to analyze and dependent on a variety of internal variations, and the collection of blood and urine for bio-fluid analysis is not convenient. However, the RII is secreted in saliva (Mednieks and Hand, 1984) and is an easily measurable biochemical indicator of environmental stress.

Numerous other salivary secretory proteins with known function may be indices of metabolic pathways and associated physiologic or pathologic functions (Mandel, 1993; Ruhl, 2012). Among those considered in this study are alpha-amylase (α-amylase) (Valdez and Fox, 1991), the proline-rich proteins (PRPs) (Mehansho et al., 1985; Carlson et al., 1991), demilune cell and parotid protein (DCPP) (Bekhor et al., 1994), and parotid secretory protein (PSP) (Owerbach and Hjörth, 1980; Ball et al., 2003). Microarray analysis of mouse salivary gene expression was carried out comparing flight with control data (Mednieks et al., 2014). By considering this initial array of secretory protein responses, a number of criteria can be established to assess the effects of the length of microgravity exposure and eventual transience or permanence of these effects upon return to Earth.

Objectives

Human and rodent salivary glands are morphologically and functionally similar. In both species, secretory proteins produced by salivary glands are stored in granules and released into saliva where they can be measured. The mouse model is, therefore, appropriate for studies of stimulated secretion. This system avoids the difficulties associated with obtaining tissue from human subjects and saliva from test animals. Our objectives, therefore, are: 1) To study the effects of weightlessness at the tissue, cell, and molecular level; 2) to identify specific molecular mechanisms affected by space travel; 3) to propose the design of a simple, economic device to measure changes in salivary proteins that can be used to monitor the physiological status of astronauts in space, or patients in Earth-based clinics.

METHODS

Animals and Space Flight / Ground Control Conditions

Thirty C57Bl/6J adult female mice, nine weeks old at launch, were housed in Animal Enclosure Modules (AEMs) and flown on the space shuttle Atlantis STS-135 mission, launched from Kennedy Space Center on July 8, 2011, with landing on July 21, 2011. Fifteen mice housed in AEMs served as ground controls. Fifteen additional control mice were housed under standard vivarium conditions (for experiment details see Gridley et al., 2013). The flight mice and AEM ground control mice were fed identical diets [NASA rodent food bars (Sun et al., 2014)] and had access to water ad libitum. The vivarium control mice were fed pelleted rodent chow. Tissues from seven of the flight mice were
obtained within 2-5 hours after landing. Tissues from the vivarium control mice were obtained one day prior to landing, and tissues from the AEM control mice were obtained 48 hours after landing. All animal procedures were approved by the Institutional Animal Care and Use Committee at the National Aeronautics and Space Administration (NASA), and conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Mice from the Bion-M1 mission were C57BL/6N specific pathogen-free males, 19-20 weeks old at launch on April 19, 2013. Samples were obtained from six flight mice between 13-16.5 hours after landing on May 19, 2013, and from eight vivarium control mice immediately afterwards. Samples were also obtained from seven asynchronous control mice, housed two months after the mission in flight habitats for 30 days under environmental conditions simulating the flight conditions, and seven asynchronous vivarium control mice. The flight mice and the asynchronous habitat control mice were fed a paste diet based on standard rodent chow, with water and casein added as a gelling agent. Vivarium control mice were fed pelleted rodent chow. Details of the experimental conditions can be found in Andreev-Andrievsky et al. (2014). All animal procedures were approved by the Institutional Animal Care and Use Committee of Moscow State University Institute of Mitoengineering and the Biomedical Ethics Commission of the Institute for Biomedical Problems, and conducted in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

**Tissue Collection**

After euthanasia, the salivary glands were excised and placed in cold phosphate buffered saline (PBS). A portion of each gland was rapidly frozen in a protease inhibitor mixture for protein assays and in RNA later or Allprotect (Qiagen, Hilden, Germany) for RNA analyses. The remaining tissues were minced and fixed in 2.5% glutaraldehyde-2% paraformaldehyde for electron microscopy, or 4% paraformaldehyde for immunocytochemistry. The samples were shipped by overnight express to the University of Connecticut Health Center.

**Secretory Proteins and Immunological Reagents**

The specific secretory proteins studied were α-amylase, PSP, DCPP, PRPs, the regulatory subunit of type II protein kinase A (PKA RII) (regulatory protein), salivary androgen binding protein alpha (SABPα), epidermal growth factor (EGF), and nerve growth factor (NGF). Antibodies used were: anti-RII (Szczepanski et al., 1998; Mednieks et al., 2008), anti-α-amylase and anti-EGF (Sigma-Aldrich, St. Louis, MO), anti-PSP (Ball et al., 1988; L. Mirels, unpublished), anti-PRPs (D.M. Carlson, unpublished), anti-DCPP and anti-SABPα (L. Mirels, unpublished), and anti-NGF (Santa Cruz Biotechnology, Dallas, TX).

**Transmission Electron Microscopy (TEM)**

Parotid glands were postfixed in 1% osmium tetroxide, treated with 1% aqueous uranyl acetate, dehydrated in ethanol, and embedded in epoxy resin following standard procedures (Hand, 1995). Thin sections were stained with uranyl acetate and lead citrate, and examined in a Hitachi H7650 TEM.

**Immunogold Labeling TEM**

Tissues were embedded in LR Gold resin (London Resin Co., Ltd., Electron Microscopy Sciences, Hatfield, PA) at -20°C. Thin sections were collected on Formvar-coated nickel grids, treated with 1% BSA/5% NGS in PBS to block nonspecific binding, and incubated with primary antibody overnight at 4°C. Bound antibodies were visualized with gold-labeled goat anti-rabbit IgG (Aurion, Electron Microscopy Sciences) or gold-labeled protein-A (BB International, Ted Pella, Redding, CA). The sections were stained with uranyl acetate and lead citrate and examined in the TEM. Digital images were taken at 10,000X using an AMT XR41C camera (Advanced Microscopy Techniques, Woburn, MA).

Quantification of immunogold labeling was done on the TEM images. Using Photoshop (CS2 v. 9.0.2 or CS4 Extended v. 11.0.2), a grid pattern with a separation between grid lines corresponding to 0.5 µm was superimposed on the image. Grid intersections lying over the organelle
of interest were counted and divided by 4 to estimate area in \( \mu \text{m}^2 \). Gold particles labeling the organelle were counted and divided by the area to give a labeling density (gold particles/\( \mu \text{m}^2 \)). Significance was assessed using ANOVA and a two-tailed T-test.

**Electrophoresis and Western Blotting**

Tissue samples were homogenized in PBS containing 12.5 mM benzamidine and mM phenylmethyl sulfonyl fluoride. The homogenate was centrifuged at 10,000xG for 10 min at 4°C and the supernatant was collected. A light microscope smear was made of the pellet to ascertain effective homogenization of cells and organelles. Concentration of total proteins in the soluble fraction was determined by densitometric analysis of Ponceau S dye intensity on slot blots, compared to those of standards of known protein concentration. The sample proteins were adjusted to identical protein concentrations per lane and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using pre-cast 10% polyacrylamide gels. The proteins were electrotransferred to nitrocellulose membranes, stained with Ponceau S, scanned, and digitized to visualize electrophoretic banding patterns. The membranes were then washed to remove the dye, blocked with a 3% milk solution in PBS - 0.01% Tween20, and incubated with specific primary antibodies. Horseradish peroxidase-conjugated secondary antibodies were applied, and the Western blot developed with 4-chloro-1-naphthol-H2O2 solution, or Vector NovaRED substrate (Vector Laboratories, Burlingame, CA). Protein concentrations and protein banding pattern profiles were determined by scanning and measuring the digitized images using ImageJ (v. 1.43u or 1.48; Public domain) software and compared to the densities of standard proteins of known concentration. A standard curve was prepared for each experiment. Average values obtained by scanning of duplicate lanes were used for comparisons among the groups. Due to the small sample size, additional Western blotting was carried out using a slot blotting method (HYBRI-SLOT™ MANIFOLD, Life Technologies Inc., Gaithersburg, MD). Duplicate samples were on the two slot blot lanes of the same membrane; antibody incubations, color development, and digitized sample analysis were carried out as described above for the electrophoretically separated protein samples.

**Microarray Analyses**

Individual tissue samples — eight control and seven flight from STS-135, and seven control and five flight from Bion-M1 — were submitted for RNA isolation and microarray analyses by Phalanx Biotech Group (San Diego/Belmont, CA) using Mouse OneArray 2 Gene Expression Profiling. All samples had high (>6) Agilent 6000 Nano Assay RNA integrity (RIN) numbers. Information about the mouse microarray can be found at http://www.phalanxbiotech.com/products/ MOA.php (accessed 5/25/15), and their gene expression service at http://www.phalanxbiotech.com/services/service_genome.php (accessed 5/25/15).

**RESULTS**

**Morphology and Immunocytochemistry**

The flight mice from both STS-135 and Bion-M1 appeared healthy upon landing. The flight mice and the AEM (habitat) control mice from STS-135 mission lost an average of 2.3 and 1.3 grams of body weight, respectively (Table 1). The flight mice and habitat control mice from the Bion-M1 mission both gained weight, 2.6 and 2.4 grams, respectively. There were no significant differences between the body weights of the flight mice and their respective habitat or vivarium controls, thus indicating a similar nutritional status.

The morphology of a parotid acinar cell of a STS-135 flight mouse is shown in Figure 2, Panel A. The cells have a basally located nucleus, abundant rough endoplasmic reticulum, a prominent Golgi complex, and numerous secretory granules stored in the apical cytoplasm. The secretory granules typically have a variable density, with a peripheral dense region and a lighter central region. Sometimes a dense area is present in the middle of the light region. The apical surface of the cells faces the central lumen of the acinus, and finger-like extensions of the lumen — intercellular canaliculi — are located along the lateral sides of the cells. Junctional complexes join the cells at the apical ends of the lateral intercellular spaces and seal the intercellular canaliculi from the intercellular...
Table 1. Body Weights of STS-135 and Bion-M1 Mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial Body Wt. (g) ± SEM (n)</th>
<th>Final Body Wt. (g) ± SEM (n)</th>
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<tbody>
<tr>
<td><strong>STS-135</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flight</td>
<td>20.7 ± 0.40 (7)</td>
<td>18.4 ± 0.52 (7)</td>
</tr>
<tr>
<td>AEM Ground Control</td>
<td>20.7 ± 0.31 (15)</td>
<td>19.4 ± 0.33 (15)</td>
</tr>
<tr>
<td>Vivarium Ground Control</td>
<td>---</td>
<td>19.7 ± 0.34 (15)</td>
</tr>
<tr>
<td><strong>Bion-M1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flight</td>
<td>26.8 ± 0.55 (6)</td>
<td>29.4 ± 1.73 (6)</td>
</tr>
<tr>
<td>Synchronous Vivarium Ground Control</td>
<td>29.2 ± 0.44 (8)</td>
<td>28.7 ± 0.30 (8)</td>
</tr>
<tr>
<td>Asynchronous Habitat Ground Control</td>
<td>27.3 ± 1.15 (7)</td>
<td>29.7 ± 0.83 (7)</td>
</tr>
<tr>
<td>Asynchronous Vivarium Ground Control</td>
<td>26.7 ± 0.78 (7)</td>
<td>28.9 ± 1.03 (7)</td>
</tr>
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</table>

Figure 1. Cyclic AMP-PKA signaling pathways of regulated exocytosis in cell compartments. Schematic representation of cell surface beta-adrenergic receptor stimulation, signal transduction, cyclic AMP second messenger cytoplasmic and nuclear response. βAR, beta-adrenergic receptor; Gs, heterotrimeric G protein; AC, adenylate cyclase; PDE, phosphodiesterase; AKAP, A-kinase anchor protein; RII, type II PKA regulatory subunit; C, PKA catalytic subunit; ATF1, activating transcription factor 1; CREB, cyclic AMP response element binding protein; CREM, cyclic AMP responsive element modulator. The large blue sphere represents a secretory granule along a path to fuse with the cell membrane and to empty its contents of secretory proteins into the lumen.
space. The basic structure of the acinar cells of STS-135 and Bion-M1 flight mice were similar, and not different from that of habitat or vivarium control mice.

There were, however, some specific changes observed in the glands of flight animals. Figure 2, Panel B, shows an intercalated duct of a STS-135 flight mouse. Intercalated ducts are the first component of the duct system connecting the acini with the striated ducts, which are the major ductal component and are involved in electrolyte reabsorption and secretion. The cells of both intercalated and striated ducts — especially in STS-135 samples, but also in Bion-M1 samples — had large apical vacuoles containing acinar secretory proteins endocytosed from the ductal lumen. The inset in Panel B shows immunogold labeling of a vacuole in a striated duct cell for PSP, an acinar secretory protein. The acinar cells of mice from both flights also showed an increased number of autophagic vacuoles and an increase in the number of apoptotic cells was observed. Panel C shows autophagic vacuoles in a Bion-M1 flight mouse containing secretory proteins and other organelles undergoing degradation. Gold particles indicating the presence of PSP, an acinar secretory protein, label the content of one autophagic vacuole and other secretory granules in the cell. Panel D illustrates two apoptotic acinar cells engulfed by a macrophage in a Bion-M1 flight mouse. The structure of the surrounding acinar cells appears normal.

Quantitative immunogold labeling was done to assess secretory protein expression in acinar cells of STS-135 and Bion-M1 flight and habitat control mice. Figure 3, Panels A and B, shows PRP labeling of secretory granules in STS-135 flight and AEM ground control samples, respectively. As shown in the micrographs, the labeling density (gold particles/μm²) of the flight granules was greater than that of the control granules, indicating greater expression of PRP in the flight mice. Panels C and D show amylase labeling in Bion-M1 flight and habitat ground control samples, respectively, demonstrating increased amylase expression in flight mice compared to habitat controls. A summary of the immunogold labeling data for flight mice, and their respective habitat controls from both STS-135 and Bion-M1, is shown in Table 2 and Figure 4. The longer flight duration of Bion-M1 resulted in some specific differences in secretory protein expression. Amylase expression was decreased in STS-135 flight samples, but increased in Bion-M1 flight samples. Expression of PSP was slightly, but significantly, increased in both flights. Expression of PRP was increased in STS-135 flight samples, but slightly decreased in Bion-M1 flight samples. On the other hand, expression of DCPP was slightly decreased in both STS-135 and Bion-M1 flight samples, and PKA RII expression was essentially unchanged from habitat controls. Table 2 also shows results of immunogold labeling of submandibular glands of Bion-M1 flight and habitat control mice. Expression of the acinar cell proteins SABPα and PRP, and the granular duct cell proteins EGF and NGF, was significantly increased in flight mice compared to the controls.

**Electrophoresis and Western Blotting**

The Bion-M1 Western blotting results showed no difference between RII in Bion-M1 flight and vivarium control mice, seen in Figure 5, Panel A. Electrophoretic banding patterns of the flight and control animals, shown in Figure 5, Panel B, were virtually identical. Similar electrophoretic banding patterns and densitometry profiles were seen in STS-135. However, as shown in the top profile of Figure 6, Panel A, the Western blotting RII band of the STS-135 flight samples was significantly smaller than the corresponding band in either vivarium or habitat controls, middle and bottom profiles, respectively. These results indicate that on the shorter, STS-135 flight the expression of RII was decreased, while in the longer Bion-M1 flight an apparent stabilization or adjustment to the microgravity environment had occurred and the RII levels were not different from those of either of the controls. Both Bion-M1 and STS-135 flight and both control samples show a significant, faster-moving fragment (Rfr) that is recognized by the anti-RII antibody. Protease inhibitors were used and samples frozen rapidly after dissection, nevertheless the faster moving component seen in the Western blot (WB) may be due to proteolysis, as may the relatively high backgrounds. Table 3 shows the ratios of the flight Rfr to total proteins are not significantly different for either the STS-135 or Bion-M1 samples compared to controls. The decrease, therefore, in RII for the STS-135...
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Figure 2. Ultrastructure of parotid glands of flight mice. Panel A, STS-135: Acinar cells have basally located nuclei [N (binucleate cells are common)], a prominent Golgi complex (G), and abundant apically located secretory granules (SG). Intercellular canaliculus (IC). Scale bar = 2 \( \mu \)m. Panel B, STS-135: Intercalated duct. The duct cells have apical vacuoles (arrows) containing material endocytosed from the lumen (L). Scale bar = 2 \( \mu \)m. The inset shows a similar vacuole in a striated duct cell immunogold labeled for PSP, an acinar secretory protein. Scale bar = 0.5 \( \mu \)m. Panel C, Bion-M1: A recently formed autophagic vacuole (AV1) containing secretory proteins labeled for PSP is present in an acinar cell. An older autophagic vacuole (AV2) contains remnants of secretory granules and other organelles, but shows little labeling. Secretory granule (SG); nucleus (N). Scale bar = 1 \( \mu \)m. Panel D, Bion-M1: Apoptotic acinar cells (Apop) phagocytosed by a macrophage (arrowheads). Myoepithelial cell (MEC). Scale bar = 2 \( \mu \)m.
flight may be due to flight conditions rather than to increased degradation. However, the ratio of RII/protein in flight samples compared with both vivarium and habitat control samples in STS-135 is significantly lower than that of Bion-M1. These results are consistent with those from immunocytochemistry experiments and microarray analyses. Figure 6, Panel B, shows that the expression of α-amylase is significantly reduced in the flight parotid, and also in the habitat control when compared to vivarium controls.

**Microarray Analyses**

Changes in the expression of selected genes associated with cyclic AMP signaling pathways in the flight animals compared with habitat control mice are shown in Figure 7. The flight duration (STS-135, 13 days; Bion-M1, 30 days) had a significant effect on the expression of most of these genes. The expression of the type II PKA regulatory subunit (Prkar2a) was decreased in STS-135 flight mice, but was near control levels in Bion-M1 flight mice. Phosphodiesterase 4a (Pde4a) also was decreased, more so in Bion-M1 flight mice than STS-135 flight mice. The two flights showed opposite effects on the expression of A-kinase anchor protein 13 (Akap13) and adrenergic receptor beta 2 (Adrb2); the former increased in STS-135 mice and decreased in Bion-M1 mice, whereas the latter decreased in STS-135 mice and increased in Bion-M1 mice. Adenylate

**Table 2. Quantitative Immunogold Labeling of STS-135 and Bion-M1 Salivary Glands (Gold Particles/µm² ± SEM).**

<table>
<thead>
<tr>
<th>Bion-M1 Parotid</th>
<th>Flight</th>
<th>Habitat</th>
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<tbody>
<tr>
<td>Amylase</td>
<td>15.3 ± 1.11∞</td>
<td>10.3 ± 0.69</td>
</tr>
<tr>
<td>RII</td>
<td>10.1 ± 0.47</td>
<td>11.3 ± 0.49</td>
</tr>
<tr>
<td>PSP</td>
<td>37.4 ± 1.17§</td>
<td>30.6 ± 1.76</td>
</tr>
<tr>
<td>PRP</td>
<td>22.5 ± 1.61</td>
<td>24.8 ± 2.08</td>
</tr>
<tr>
<td>DCPP</td>
<td>23.1 ± 0.95</td>
<td>25.2 ± 1.27</td>
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<table>
<thead>
<tr>
<th>STS-135 Parotid</th>
<th>Flight</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase</td>
<td>13.9 ± 0.58§</td>
<td>17.0 ± 0.76</td>
</tr>
<tr>
<td>RII</td>
<td>11.9 ± 0.36</td>
<td>12.1 ± 0.47</td>
</tr>
<tr>
<td>PSP</td>
<td>16.5 ± 0.35*</td>
<td>15.2 ± 0.51</td>
</tr>
<tr>
<td>PRP</td>
<td>21.2 ± 0.87∞</td>
<td>17.2 ± 0.67</td>
</tr>
<tr>
<td>DCPP</td>
<td>12.5 ± 0.45</td>
<td>14.1 ± 0.75</td>
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<table>
<thead>
<tr>
<th>Bion-M1 Submandibular</th>
<th>Flight</th>
<th>Habitat</th>
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<tbody>
<tr>
<td>SABPα</td>
<td>5.30 ± 0.27∞</td>
<td>2.40 ± 0.13</td>
</tr>
<tr>
<td>PRP</td>
<td>6.36 ± 0.21∞</td>
<td>4.73 ± 0.35</td>
</tr>
<tr>
<td>EGF</td>
<td>32.5 ± 0.60∞</td>
<td>20.4 ± 1.09</td>
</tr>
<tr>
<td>NGF</td>
<td>8.78 ± 0.41∞</td>
<td>5.52 ± 0.37</td>
</tr>
</tbody>
</table>

Significantly different from habitat: * p<0.05; § p<0.01; ∞ p<0.001
Figure 3. Immunogold labeling of parotid acinar cell secretory granules. Panel A, STS-135: Flight, PRPs. Panel B, STS-135: AEM ground control, PRPs. Panel C, Bion-M1: Flight, amylase. Panel D, Bion-M1: Habitat ground control, amylase. Intercellular canaliculus (IC); mitochondrion (M). Scale bar = 0.5 µm.
Figure 4. Morphometric analysis of immunogold labeling of parotid secretory proteins. Percent change in STS-135 (light bars) and Bion-M1 (dark bars) flight mice from habitat controls.

Figure 5. Electrophoresis, Western blotting, and densitometry of parotid proteins. Panel A, Bion-M1 control and flight parotid soluble proteins. The lanes are molecular size marker (M), flight (F), and vivarium control (C). The two bands on the Western blot (WB) are at the mobility of RII and an RII fragment ($R_{fr}$), respectively. Panel B, Densitometry of the banding pattern profiles. Vivarium control (light gray curve) superimposed with the flight (darker curve) protein electrophoretic pattern. The ordinate shows integrated density (ID) values and the abscissa shows the calculated molecular size in kilo Dalton (kD) units.
Figure 6. Densitometric analysis of polyacrylamide gel electrophoretic protein separation and anti-RII and anti-α-amylase reactivity in STS-135 mice. Panel A: Top panel, flight; middle panel, vivarium; and bottom panel, habitat parotid gland samples. The lighter curve represents the protein profile; the shaded area is the reactivity to anti-RII antibody. The ordinate axes are integrated density values of the proteins on the left hand axis, and the integrated density values for RII and the reactivity of RII fragment (Rfr) on the right hand axis. Panel B shows parotid α-amylase Western blotting. The ordinate shows integrated density, and the error bars show ± 5% error. F, flight; C1, vivarium control; C2, habitat control.
Figure 7. Microarray analyses of selected parotid genes. The ordinate shows the log2 values of the ratio of the means of the normalized signal intensities determined for each gene for flight vs. habitat control. Prkar2a, protein kinase, cAMP dependent regulatory, type II alpha; Akap13, A-kinase anchor protein 13; Pde4a, phosphodiesterase 4a, cAMP specific; Adrb2, adrenergic receptor, beta 2; Adcy3, adenylate cyclase 3; Amy1, amylase 1, salivary.

Table 3. Ratios of RII to Total Protein of STS-135 and Bion-M1 PAGE.

<table>
<thead>
<tr>
<th></th>
<th>STS-135</th>
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<tbody>
<tr>
<td></td>
<td>F</td>
<td>C1</td>
<td>C2</td>
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<tr>
<td>PAGE</td>
<td>70.8</td>
<td>69.28</td>
<td>69.99</td>
</tr>
<tr>
<td>RII</td>
<td>5.16</td>
<td>41.43</td>
<td>24.24</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.073</td>
<td>0.60</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>Bion-M1</td>
<td></td>
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</tr>
<tr>
<td>PAGE</td>
<td>153.6</td>
<td>129.0</td>
<td>---</td>
</tr>
<tr>
<td>RII</td>
<td>21.7</td>
<td>16.26</td>
<td>---</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.14</td>
<td>0.13</td>
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Values are integrated density measurement for total protein and RII Western blots when flight (F) samples are compared to those of vivarium controls (C1) and habitat controls (C2).
Table 4. Microarray Analyses of Secretory Protein Gene Expression.

<table>
<thead>
<tr>
<th>Gene*</th>
<th>STS-135 Log2 F/H</th>
<th>STS-135 Fold Change F/H</th>
<th>Bion-M1 Log2 F/H</th>
<th>Bion-M1 Fold Change F/H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amy1</td>
<td>0.030</td>
<td>1.02</td>
<td>0.169*</td>
<td>1.124</td>
</tr>
<tr>
<td>Bpifa2</td>
<td>0.241§</td>
<td>1.18</td>
<td>0.170*</td>
<td>1.13</td>
</tr>
<tr>
<td>Lpo</td>
<td>0.070</td>
<td>1.05</td>
<td>0.160*</td>
<td>1.18</td>
</tr>
<tr>
<td>Lyz1</td>
<td>0.317</td>
<td>1.25</td>
<td>0.898∞</td>
<td>1.86</td>
</tr>
<tr>
<td>Pip</td>
<td>0.228*</td>
<td>1.17</td>
<td>0.169*</td>
<td>1.13</td>
</tr>
<tr>
<td>Prpmp5</td>
<td>-0.161§</td>
<td>0.894</td>
<td>-0.861∞</td>
<td>0.550</td>
</tr>
</tbody>
</table>

* Amy1, amylase 1, salivary; Bpifa2, BPI fold containing family A, member 2 (parotid secretory protein); Lpo, lactoperoxidase; Lyz1, lysozyme 1; Pip, prolactin induced protein; Prpmp5, proline-rich protein MP5

b flight/habitat control

*, p<0.05; §, p<0.01; ∞, p<0.001

cyclase 3 (Adcy3) expression was increased in STS-135 flight mice, but was similar to that of controls in Bion-M1 flight mice. Expression of amylase 1 (Amy1), which is regulated by catecholamine and insulin signaling, was slightly increased in mice from both flights.

Table 4 shows changes in the expression of secretory protein genes in flight animals compared with habitat controls. The increased flight duration of Bion-M1 had opposite effects on the expression of two secretory protein genes, increasing lysozyme (Lyz1) and decreasing PRP (Prpmp5). In contrast, the expression of the genes for parotid secretory protein (Bpifa2), lactoperoxidase (Lpo), and prolactin inducible protein (Pip) showed only small changes from controls and did not differ between the two flights. These results show individual secretory protein expression responds to travel in space, and the microgravity environment has specific gene regulatory effects on secretory and signaling pathway proteins. Additionally, there is a flight duration dependent component where initial responses may show opposite changes or readjust to basal levels.

DISCUSSION

Space travel is ideal for studying the effects of an environment that cannot be effectively reproduced on Earth. The animal samples obtained from the space missions are unique and the experiments have to be precise and unambiguous. For that reason, we have selected three different experimental approaches (electron microscope based, biochemical, and molecular) to ensure the experimental results are verified by using several methods and the likelihood of technical variability is significantly reduced. The expression of secretory proteins is affected by a variety of physiological, pathological and, as is the case in microgravity, environmental factors. Secretory proteins themselves are important indices of the various functions of an organism. Proteins secreted into saliva serve as markers of the various cell types of the gland and the condition of the oral cavity and the proximal portion of the alimentary tract. In addition, secretory proteins representing other physiologic functions are present in saliva and can serve as
The proteins selected for study in these experiments are representative of digestive functions [α-amylase (Valdez and Fox, 1991)]; calcium and polyphenol binding [PRPs (Bennick, 1982; Bennick, 2002)]; antimicrobial [PSP (Geetha et al., 2003) and PRPs (Robinovitch et al., 2001)]; microbial binding activity [α-amylase (Scannapieco et al., 1993), PSP (Robinovitch et al., 1997), and DCPP (Ambatipudi et al., 2010)]; and regulatory activity [PKA RII (Daniel et al., 1998; Gold et al., 2013)]. In the parotid gland, α-amylase, PRPs, and PSP are products of acinar cells; DCPP is secreted by intercalated duct cells; and PKA RII is present in acinar (Mednieks et al., 1987) and striated duct cells (Piludu et al., 2002), and secreted by acinar cells (Mednieks and Hand, 1984).

It is possible differences in diet may have an effect on salivary glands. Parotid gland atrophy, gland weight, and enzyme activity decrease in rats fed liquid diet (Hall and Schneyer, 1964; Johnson, 1982; Zelles et al., 1989; Takahashi et al., 2012). Additionally, acinar cells exhibit increased numbers of autophagic vacuoles and increased apoptosis (Hand and Ho, 1981; Takahashi et al., 2012). However, in both STS-135 and Bion-M1 flight mice there were significant differences in protein expression, as well as an increase in autophagy and apoptosis compared to their respective habitat control glands, suggesting that regardless of diet, these processes were affected by microgravity. The presence of secretory granules and secretory proteins in autophagic vacuoles suggests granule exocytosis may have decreased, with prolonged retention of granules within the cytoplasm. Moreover, the increase in amylase protein shown by immunogold labeling in the Bion-M1 flight mice is opposite than expected for liquid diet (Zelles et al., 1989). Interestingly, liquid diet feeding has no apparent effect on rat submandibular and sublingual glands (Takahashi et al., 2014).

Vacuoles containing acinar secretory proteins in the apical cytoplasm of intercalated and striated duct cells of flight mice indicate endocytosis of proteins from the lumen. Whether this is a result of altered duct cell function or altered protein structure is unknown. Similar acinar protein containing vacuoles were seen in diabetic rats (Lotti and Hand, 1988), and duct cells avidly endocytose foreign proteins introduced retrogradely into the duct system (Hand et al., 1987).

The results from Western blotting are consistent with those of immunocytochemistry and microarray analyses. As reported previously (Mednieks et al., 2014), an important change occurs in parotid RII during short-term flights, where secretory protein expression is initially affected by microgravity. In the case of RII, it appears to return to basal levels during the longer Bion-M1 flight. This apparent stabilization may be a homeostatic response, at least in the catecholamine regulated secretory pathway. The mechanism for the stabilization might be related to the decrease in PDE and an increase in the expression of the adrenergic receptor, thus pushing the reaction to counter a possible decrease of RII expression. The submandibular gland, however, shows a much smaller component of RII and it apparently is unaffected (data not shown) by microgravity conditions. Parotid α-amylase initially is decreased but during the longer flight returns to baseline and, subsequently, higher levels than controls.

The microarray analyses confirm microgravity affects the expression of signaling pathway and secretory protein genes in salivary glands. The effects on expression of some genes were similar in both flights (e.g., Prkar2a and Pde4a were both decreased), although Prkar2a showed a return to baseline in the longer flight, while Pde4a was further decreased. The flight duration affected some genes in an opposite manner (e.g., the expression of Akap13 was increased in the shorter flight but decreased in the longer flight), whereas the expression pattern of Adrb2 was the reverse.

Although the microarray results revealed significant changes in the expression of several secretory protein genes, only two, Lyz1 and Prpmp5, showed a substantial fold change in expression with the longer Bion-M1 flight. Interestingly, these changes were in opposite directions — the expression of Lyz1 being increased and Prpmp5 being decreased. The changes observed by the three analytical approaches were not always coincident or of corresponding magnitude. For example, immunogold labeling showed a significant
increase in PRP expression on STS-135 and a small decrease on Bion-M1, whereas the microarray results indicated a small decrease in Prpmp5 expression on STS-135 and a large decrease on Bion-M1. Similarly, α-amylase showed an almost 50% increase by immunogold labeling of Bion-M1 samples, but only a 12% increase in Amy1 expression by microarray and an apparent decrease by Western blotting. It is apparent that secretory protein expression under microgravity conditions is individually affected, rather than an overall response. Whether these differences result from different rates of transcription and translation, or different rates of protein and mRNA turnover, is unknown, and further investigation is required for a better understanding.

Other differences between the STS-135 and Bion-M1 missions included the age and sex of the mice. Both male and female mice are considered sexually mature at about eight weeks of age (Fox et al., 2007). The parotid gland reaches its definitive morphology by about four weeks of age (Ribeiro Castro et al., 2006). The expression of secretory proteins during mouse parotid gland development has not been thoroughly documented; however, in the rat, amylase, DNase, and RNase activities are essentially at adult levels by 40 days of age (Redman and Sreebny, 1971). Few, if any, differences have been described between male and female parotid glands; therefore, the age and sex differences of the mice on the two flights are not expected to have effects on secretory protein expression. There are significant morphologic, biochemical, and functional differences between male and female submandibular glands (Pinkstaff, 1998), but in this report we present only the results from the Bion-M1 mission.

Although the effects of different modes of post-flight transport are not known, recent findings indicate the difference in tissue harvesting times between the STS-135 and Bion-M1 missions were unlikely to have had a significant effect on our results. Studies of microvascular vasoconstrictor responses of vessels from mice allowed to recover for one day after landing (STS-133) were qualitatively similar to those of vessels obtained from mice within two to five hours after landing (STS-131) (Behnke et al., 2013). At five and seven days after landing, vasoconstrictor responses were similar to those of vessels of ground control mice. Preliminary data (unpublished) on the expression of selected parotid proteins from STS-133 mice show values similar to those of STS-131 mice, and a recovery to ground control values by five days after landing.

Thus, the major effects on changes in secretory protein expression observed in the present study are likely to be due to microgravity. Early hypergravity (1.7xg) and adrenergic stimulation experiments showed no short duration effect (Mednieks et al., 1998). Catecholamine analog (isoproterenol injections) stimulation showed a short and transient initial response, as might occur during lift-off or re-entry, but extended stimulation showed a more lasting and measurable effect (Mednieks and Hand, 1984).

As the salivary glands are an advantageous experimental system of the mouse model, so their secretory proteins are convenient for study and eventually for application to measuring human responses (Mednieks et al., 1994). In this study three approaches — immunocytochemical, biochemical, and molecular — were employed to determine the effects of microgravity. The findings of these experiments were consistent in the changes that were observed. The results indicated the effects may be time-dependent. Namely, the shorter flight showed an effect on the major salivary proteins that appeared to be diminished after the longer Bion-M1 mission. The longer exposure to microgravity may result in a homeostatic response (e.g., Selye, 1973).

SUMMARY AND FUTURE GOALS

Travel in space has an effect on oral tissues and specific secretory proteins. Tissue changes appear to involve autophagic, apoptotic, and endocytic activity. Molecular changes — especially those associated with catecholamine hormone responses — are affected, but tend to return to basal levels on longer flights.

Future goals are to elucidate specific metabolic pathways involved in altered physiologic functions and to use secretory proteins as markers for these events. In progress is the design of an economical, practical testing device for measuring markers in a bio-fluid for
use in space travel and adapted for clinical use on Earth.

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